Structural Studies of Neutrophil Gelatinase Associated Lipocalin (NGAL) and the MHC Class I Homolog MIC-A

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INTRODUCTION

Human Neutrophil Gelatinase Associated Lipocalin (NGAL) was originally identified as a component, along with gelatinase (MMP-9), of a disulfide-linked heterodimer secreted by neutrophils **[1]**. NGAL can also be secreted from neutrophils as both a 25 kD monomer and as a 46 kD disulfide-linked dimer in the absence of associated gelatinase **[1, 2]**. Neutrophils in the colonic mucosa do not actively synthesize NGAL; rather, the protein is produced in immature neutrophil precursors in the bone marrow and stored in specific granules for subsequent release. Unlike other lipocalins, NGAL shows no affinity for retinoic acid, but does bind the N-formylated tripeptides fMet-Leu-Phe (fMLF) and fNorleucine-Leu-Phe (fNLF) which are active in bacterial chemotaxis **[3]**. The dissociation constant of NGAL and photo-labeled fMLF is 145 nM; competition assays yield a dissociation constant for fMLF of between 30 and 60 nM **[4]**. NGAL synthesis is highly induced in epithelial cells in both inflammatory and neoplastic colorectal diseases **[5]**. Its expression is restricted to the affected tissue only. NGAL may also bind other lipophilic mediators of inflammatory responses, notably platelet activating factor and leukotriene B_4 **[5]**. The murine analogue of NGAL, 24p3, was originally identified in a screen for genes overexpressed during a SV40-induced mitotic reaction **[6]**. 24p3 expression is also amplified by an autocrine mechanism in response to dexamethasone or retinoic acid **[7]**. The synthesis of 24p3 has also been shown to be induced in cultured macrophages by lipopolysaccharide **[8]**. The rat analogue of NGAL, *neu*-related lipocalin (NRL) is specifically overexpressed in *neu*-initiated rat mammary carcinomas **[9]**.

MIC-A: Responses by the cellular component of the acquired immune system are mediated by the specific recognition of foreign peptides bound to Major Histocompatibility Complex (MHC) proteins by $\alpha\beta$ T cell receptors (TCRs) **[10]**. A second class of T cells, those bearing $\gamma\delta$ TCRs, are proposed to mediate antimicrobial responses and regulate innate and acquired immunity **[11]**. The rules governing the interactions between $\gamma\delta$ TCRs and their ligands, while currently undefined, are believed to be distinct from those directing the interaction between $\alpha\beta$ TCRs and their ligands. One subset of $\gamma\delta$ T cells, those expressing $V_\delta1$ TCRs, recognize the distant MHC class I homologs MIC-A and MIC-B **[12-16]**. Through this interaction, MIC-A is proposed to function as a stress-induced self antigen on gastric epithelial cells **[12, 13]**. Unlike MHC class I proteins, MIC-A requires neither peptide nor β_2-microglobulin (β_2-m) for stability or cell-surface expression **[17-19]**. We propose to determine the crystal structure of a soluble fragment of human MIC-A in order provide a structural context for understanding $\gamma\delta$ TCR-mediated recognition. The "classical" or class Ia MHC proteins (HLA-A, B and C) are integral-membrane, heterodimeric proteins that are polymorphic and ubiquitously expressed. This sequence polymorphism reflects the ability of class Ia molecules to bind to a variety of antigenic peptides and TCRs. MIC-A and MIC-B, conserved in most mammals except rodents, are homologous to MHC class I proteins.

Unlike MHC class Ia proteins, the expression of MIC proteins is essentially restricted to gut epithelium under the control of heat-shock promoter elements. While MIC-A and MIC-B proteins are quite similar to each other (84% identical), they have diverged significantly from the MHC class I family as a whole **[20]**. A series of primate MIC sequences is now available **[20]**. The promiscuous binding of $V_\delta1+$ IELs to MIC targets is demonstrated by the uniform cytolytic response of human intestinal epithelial tumor-derived $V_\delta1+$ clones (bearing $V_\gamma1.8/V_\delta1/J_\delta1$ $\gamma\delta$ TCRs) to cells transfected with any of these MIC proteins **[20]**. These results have been interpreted as evidence for a single, conserved recognition site on MIC proteins. This interpretation contrasts the situation in class I proteins, where sequence *polymorphisms* map to peptide and TCR binding sites.

STUDIES CONDUCTED AT THE ADVANCED LIGHT SOURCE AND FHCRC

We have grown crystals of dimeric NGAL at pH = 4.5 (space group: $P4_32_12$) and monomeric NGAL at pH = 7.0 (space group: $P4_12_12$). The dimeric NGAL data were phased with PIP SIRAS phases refined with SHARP/SOLOMON. The monomeric data were phased by molecular replacement with a partial model of an NGAL half-dimer using EPMR. We are currently refining both structures (some loops and side-chains differ in conformation between the various structures). We have also collected data from NGAL co-crystallized with one putative ligand: fMLF. A bound sulphate is seen in the binding site in the monomeric NGAL structure. Interestingly, there is density in the un-soaked NGAL dimer structure that possibly corresponds to a natural ligand carried along during the purification. The density is currently consistent with a partially-occupied fatty acid (most likely palmitate), or possibly some related compound. We are currently conducting a series of mass-spec experiments to further characterize this natural ligand, but several experimental difficulties complicate this analysis. Diffraction data have been collected at ALS with the intent of extending the resolution for NGAL crystallized as is or in the presence of a variety of potential ligands.

We have grown crystals of human MIC-A at low (pH = 5.5) that diffract to a resolution of 2.8Å (space group: $F4_132$). These data were phased by MIR. The initial experimental maps were high quality, likely due to the high solvent content of the crystals (≈78%) which aided solvent-flattening. We are currently refining the structure of MIC-A against these data. Preliminary results suggest that the structure of MIC-A is the most highly diverged of the MHC class I family, with many differences in loop structure and the inter-domain relationship. One striking feature is that a stretch of ten residues in the middle of one of the helices that define the peptide-binding groove is completely disordered in our structure. We have been able to transfer crystals of MIC-A grown at pH = 5.5 to pH = 7.0 and pH = 8.0. We have been able to stabilize the crystals at these pH's by rapid cryo-preservation. We have collected diffraction data from these transferred crystals, though the resolution suffers as a result of the transfer. Preliminary results suggest that the loop disordered at pH = 5.5 partially orders progressively at the higher pH's. In solution, the protein is monodisperse at pH's ranging from 5.5 to 7.4, but begins to aggregate at pH = 8.0. Preliminary measurements of the melting temperature (T_m) of MIC-A by CD spectroscopy suggest that MIC-A has an unusually low T_m (48°) that increases to a more reasonable value at pH = 5.5 (56°). The range of this pH change suggests that protonation of histidine residues may play a role in stabilizing MIC-A. Diffraction data have been collected at ALS with the intent of extending the resolution for MIC-A crystals at pH = 5.5, 7.0 and 8.0.

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